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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No. 53466/200

In re patent application of
Tadamitsu KISHIMOTO et al.
Serial No. 08/817,084
Filed: April 7, 1997

Group Art Unit: 1816
Examiner: F. Vandervegt

For: CHRONIC RHEUMATOID ARTHRITIS THERAPY CONTAINING IL-6 ANTAGONIST AS EFFECTIVE COMPONENT

CLAIM FOR CONVENTION PRIORITY

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

The benefit of the filing date of the following prior foreign application filed in the following foreign country is hereby requested, and the right of priority provided in 35 U.S.C. § 119 is hereby claimed:

Japan Patent Application No. 5-180303 filed 07/21/93.

A certified copy of said original foreign application has been filed in U.S. Application Serial No. 08/265,520, and a copy of the English translation of thi document is attached.

Respectfully submitted,

January 22, 1998
Date

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[NAME OF DOCUMENT] APPLICATION FOR PATENT

[REFERENCE NUMBER] 934106

[SPECIFIC MATTER] Patent Application Enjoying Article 30, Paragraph 1 of the Japanese Patent Law

[DATE SUBMITTED] July 21, 1993

[DESTINATION] To Commissioner, Patent Office:
Mr. Wataru Aso

[INTERNATIONAL PATENT CLASSIFICATION] A61K 39/00

[TITLE OF THE INVENTION] A Composition for the Inhibition of Bone Resorption Comprising Interleukin-6 Receptor Antibody

[NUMBER OF CLAIMS] 3

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[Name of Article] Specification 1
[Name of Article] Drawing 1
[Name of Article] Abstract 1

[NEED FOR PROOF] Yes

[NAME OF DOCUMENT] SPECIFICATION

[TITLE OF INVENTION] A Composition for the Inhibition of Bone Resorption Comprising Interleukin-6 Receptor Antibody

[SCOPE OF CLAIM FOR PATENT]

[CLAIM 1] A pharmaceutical composition for the inhibition of bone resorption comprising anti-interleukin-6 receptor antibody as an effective component.

[CLAIM 2] A pharmaceutical composition according to claim 1 wherein the antibody to interleukin-6 receptor is derived from mouse.

[CLAIM 3] A pharmaceutical composition according to claim 1 or 2, wherein the interleukin-6 receptor is derived from mouse.

[DETAILED DESCRIPTION OF INVENTION]

[0001]

[Field of Utilization in Industry]

The present invention relates to an inhibitor of bone resorption comprising an antibody directed to the interleukin-6 receptor ('anti-IL-6R antibody' hereafter) as an effective ingredient.

[0002]

[Prior Art and Problems to be Solved by the Invention]

Bone tissue is renewed in a precisely balanced process known as bone remodeling (whereby osteogenesis) by osteoblasts, and bone resorption by osteoclasts, while preserving bone shape. This tissue renewal plays an important role in the maintenance of function of an organism.

However, once the balance between osteogenesis and bone resorption is disrupted, bone tissue enters an abnormal state

that results various diseases.

[0003]

The osteoclasts responsible for bone resorption are derived from macrophage-lineage cells, which differentiate into osteoclasts in a process dependent on intercellular contact with osteoblasts. Factors which promote this process are called bone-resorbing factors, such as an active form of vitamin D_3, parathyroid hormone, interleukin-1 (IL-1), prostaglandins, and others.

It is known that exogenous administration of an excess of these bone-resorbing factors stimulates in vivo the activation and proliferation of osteoclasts, promoting bone resorption (for examples, see Igaku no Ayumi 165: 572 - 576, 1993).

[0004]

Interleukin-6 (IL-6) was initially thought to be a cytokine which promotes the growth of B-lymphocyte lineage cells, but subsequent work has demonstrated that IL-6 can influence the immune system by inducing the maturation of T-lymphocytes as well (for an example see Lotz, et al., J. Exp. Immunol. 18: 1253 - 1258, 1988). Furthermore, it was suggested that IL-6 can affect bone tissue, because the cytokine helps to regulate the proliferation and differentiation of hematopoietic stem cells and because IL-6 is produced by a variety of cell types.

[0005]

Ishimi, et al. (J. Immunol. 145: 3297 - 3803, 1990) reported that mouse osteoblasts produce a large amount of IL-6 when stimulated with bone resorbing factors such as IL-1 and tumor necrosis factor (TNF), and as a result in vitro bone resorption is induced. In addition, it is known that IL-6 exhibits bone resorbing activity in mouse calvaria (J. Immunol. 145: 3297 - 3803, 1990). In addition, it is known

that IL-6 stimulates bone resorption in fetal mouse metacarpals (J. Immunol., 145: 3297 - 3803, 1990). It has also been reported that nude mice transplanted with CHO cells transfected with the IL-6 gene suffered from hypercalcemia (Endocrinology, 128: 2657 - 2659, 1991).

[0006]

However, Al-Humidan, et al. (J. Bone Miner. Res. 6: 3 - 8, 1991) and Barton, et al. (Cytokine 2: 217 - 220, 1990) reported that resorption of mouse calvaria does not occur with IL-6 exposure. Littlewood, et al. (J. Bone Miner. Res. 6: 141 - 148, 1991) reported that IL-6 produced by osteoblasts does not influence the growth and differentiation of osteoblasts, because osteoblasts stimulated with PTH, lipopolysaccharide (LPS), TNFα or IL-1 produce IL-6, but IL-6 does not provide any action affecting the differentiation and growth of osteoblastoid cells. In addition, Littlewood, et al. (Endocrinology 129: 1513 - 1520, 1991) reported that, although the production of IL-6 by osteoblasts was increased by PTH stimulation, the production of messenger RNA (mRNA) coding for IL-6 receptor (IL-6R) was not.

[0007]

Therefore, according to the literature, the relationship between IL-6 and bone resorption depends on the materials and experimental systems used (for more detail see a review by Roadman, J. Bone Miner. Res. 7: 475 - 478, 1992).

Recently, Manolagas, et al. reported that estrogen inhibits the in vitro production of IL-6, and that both estrogen and anti-IL-6 antibodies inhibit the proliferation of osteoclasts induced by ovariectomizing mice (J. Clin. Invest. 89: 883 - 891, 1992). However, this report does not provide any data as to whether or not the increased osteoclast population has been activated to mature cells) to the extent sufficient for bone resorption activity. Therefore, it is still not clear whether IL-6 is directly involved in bone

loss.

[0008]

[Means for Solving the Problems]

The present inventors examined the role of IL-6 in in vitro bone resorption. Hypercalcemia and the promotion of formation of osteoclasts were not observed in transgenic mice in which the human IL-6 gene was obligatorily expressed. Therefore, the inventors researched the possible involvement of factors other than IL-6. As a result, the inventors found that, although IL-6 alone did not stimulate osteoclast formation substantially, IL-6 has a strong osteoclast formation action in the presence of soluble IL-6R. The osteoclast formation induced by IL-6 and soluble IL-6R together is inhibited in the presence of anti-IL-6R antibody.

Accordingly, the present invention provides a therapeutic composition which is an inhibitor of bone resorption, comprising an anti-interleukin-6 receptor antibody (anti-IL-6R antibody).

[0009]

[Detailed Description]

According to the present invention, an anti-IL-6R antibody showing an inhibitory effect on bone resorption can be used regardless of the source of the antibody and/or antigen used to generate that antibody (i.e. different animal species).

Polyclonal or monoclonal anti-IL-6R antibodies can be obtained according to known means. For example, anti-human IL-6R polyclonal antibodies can be obtained by introducing a known IL-6R gene as disclosed in EP325474A into a known expression vector system, expressing the IL-6R protein in appropriate host cells, and purifying it from the host cells or the culture supernatant, and then using the protein as a sensitizing antigen to immunize a mammal.

[0010]

Anti-mouse IL-6R antibody can be obtained using a gene sequence disclosed in Japanese Patent Publication No. 3-155795, using the procedure described above.

There are two types of IL-6R, one expressed on the cell membrane and another which has been liberated from the cell membrane (soluble IL-6R, hereafter), the soluble IL-6R being different from IL-6R bound to the cell membrane in that the cell membrane-bound IL-6 protein comprises an extracellular domain, a transmembrane domain and a cytoplasmic domain, while the soluble IL-6R lacks the transmembrane and cytoplasmic domains.

[0011]

Antigens used for preparing the anti-IL-6R antibody of the invention include sIL-6R, which can be obtained according to conventional procedures (see, for example, Japanese Patent Publication No. 4-98800).

A monoclonal sIL-6R antibody can be obtained by immunizing a mammal with sIL-6R protein as a sensitizing antigen, fusing that mammal's plasma cells (immune cells) with myeloma cells of a mammal such as mouse, cloning the resulting fused cells (hybridomas), selecting a clone which neutralizes the activity of IL-6R, and culturing the clone to recover the desired antibody.

[0012]

Although mammals to be immunized with a sensitizing antigen are not specifically limited, preferably the mammalian plasma cells are compatible with common myeloma fusion partners which are generally of mouse, rat, hamster, etc. origin.

The myeloma cells of mammals used as parent cells to be fused with said immune cells can be from various known cell

lines, for example P3 (P3X63Ag8.653) (J. Immunol. 123: 1548, 1978), p3-UI (Current Topics in Microbiology and Immunology, 81: 1 - 7, 1978), NS-1 (Eur. J. Immunol., 6: 511 - 519, 1976), MPC-11 (Cell, 8: 405 - 415, 1976), SP2/0 (Nature, 276, 269 - 270, 1978), FO (J. Immunol. Meth., 35: 1 - 21, 1980), S194 (J. Exp. Med., 148: 313 - 323, 1978) R210 (Nature, 277: 131 - 133, 1979), etc. are preferably used.

[0013]

Cell fusion of said immune cells and myeloma cells can be carried out according to conventional procedure, such as that of Milstein, et al. (Methods Enzymol., 73: 3 - 46, 1981).

Specifically, for example, the cell fusion can be carried out in a conventional nutrient medium in the presence of fusion-promoting agents, for example, polyethylene glycol (PEG), Sendai virus (HVJ), etc. Optionally, an additive such as dimethylsulfoxide, etc. can be used to increase the fusion efficiency.

[0014]

The ratio of immune cells to myeloma cells in the fusion is, for example, 1 - 10 immune cells per a myeloma cell. As a medium for cell fusion, for example, RPMI-1640 is preferable for the growth of said myeloma cell lines. MEM medium and other conventional media used for similar cell culturing may be used. In addition, a serum supplement such as fetal calf serum (FCS) may be used in combination.

[0015]

The cell fusion is carried out by mixing predetermined amounts of said immune cells and myeloma cells in said medium and adding PEG solution to the resulting mixture (for example, PEG having an average molecular weight of about 1,000 to 6,000), to make a concentration of about 30 to 60% (w/v) PEG. Next, additional medium is sequentially added to the mixture, and the mixture is centrifuged to remove the supernatant.

This procedure is repeated until the desired hybridoma is formed.

[0016]

The hybridoma is selected by culturing in, for example, HAT medium (a medium which contains hypoxanthine, aminopterin and thymidine). The culture by HAT medium is continued for sufficient time, usually several days to several weeks, to kill cells other than hybridomas (non-fused cells). Next, screening and separating hybridoma clones producing a desired monoclonal antibody are carried out according to conventional limiting dilution methods.

[0017]

In addition, in cases where the antibody obtained is other than human, it can be converted to a reshaped human antibody in which its antigen recognizing regions (CDRs) are maintained, but framework regions (FRs) and constant regions are replaced with the corresponding regions of a human antibody. As an example, reshaped anti-sIL-6R antibodies are described in PCT Patent Publication WO 92/19759.

[0018]

For the anti-sIL-6R antibody used in the present invention, the species from which the sIL-6R antigen and the antibody-producing hybridoma are derived can be chosen with respect to the species of the subject animal to which the antibody is to be administered. Generally, when the antibody is to be administered to a human, a human cell-derived antibody to human sIL-6R is preferred, and when the antibody is to be administered to a mouse, a mouse cell-derived antibody to mouse sIL-6R is a preferred, although any combination of species may be used so long as the combination is clinically acceptable.

[0019]

The therapeutic composition of the present invention for

bone resorption is effective for the treatment of various diseases of bone metabolism, where an effective treatment would be the inhibition of IL-6R-dependent bone resorption. These diseases include osteoporosis, chronic arthritic rheumatism, multiple myeloma, tumor associated hypercalcemia, renal osteodystrophy, Paget's disease, osteosarcoma and bone tumor metastasis.

[0020]

The composition of the present invention can be administered through usual routes. For example, it can be systemically administered for example orally as a tablet or capsule or by injection or locally for example parenterally. In addition, the invention may be a pharmaceutical composition or a kit comprising at least one pharmaceutical carrier or diluting agent.

[0021]

The dosage to be administered varies depending on the severity of disease and administration route. Generally for humans, the daily dose is about 25 to 100 μg/patient up to four times per day, although other administration dosages and schedules can be used.

Note that there is no report which shows that an anti-IL-6R antibody is toxic.

[0022]

[EXAMPLES]

Now the present invention is explained in further detail by examples.

Reference Example 1 Preparation of mouse IL-6

A double-stranded cDNA was synthesized from about 10^8 P388D1 (IL-1) cells (Nordan, et al., Science 233: 566 - 569, 1986; Bazin, et al., J. Immunol. 139: 780 - 787, 1987)

according to random priming using a Fast Track™ (Invitrogen).

[0023]

In addition, an oligomer complementary to the sequence 21 - 43 of the mouse IL-6 gene (including the translation start codon (34th ATG) and having a restriction enzyme Bam H1 recognition site at the 5' terminus) and an oligomer complementary to the sequence 658 - 683 of the mouse IL-6 gene (containing the stop codon (667th TAG) and having a restriction enzyme Bam H1 recognition site at the 3' terminus) were synthesized to be used as primers.

[0024]

Using these primers and a Gene Amp kit (Takara Shuzo), PCR was carried out on a DNA Thermo Cycler (Takara Shuzo) with the following protocol: 50 cycles of 94°C for 1 minute, 50°C for two minutes and 73°C for three minutes. The amplified DNA fragment (0.66 kb) was purified by electrophoresis on low-melting agarose gel after treatment with Bam H1, then introduced into a pUC19 vector for sub-cloning. The resulting vector was cleaved with Bam H1 to obtain the mouse IL-6 gene, which was then introduced into a Bam H1 treated pdR. 50 nM MTX resistant CHO cells were selected and used for the following transfection.

[0025]

Reference Example 2 Preparation of mouse soluble anti-sIL-6R antibody

(1) An anti-mouse soluble IL-6 receptor antibody RS12 was obtained by a method of Saito et al. (J. Immunol., 147: 168 - 173 (1991). This antibody was of IgG2a subclass.

[0026]

(2) CHO cells producing a mouse soluble IL-6 receptor as disclosed in said Saito et al. reference were cultured in IMDM medium containing 10% FCS, and the supernatant was purified

using an affi[redacted]y column comprising RS12 a[redacted]body immobilized to Affigel 10 (Biorad). 50 μg of mouse soluble IL-6 receptor thus obtained was mixed with Freund's complete adjuvant, and subcutaneously inoculated to the abdomen of Wister rats (Nippon Chales River). After 2 weeks, the rats were boosted with Freund's incomplete adjuvant. After 45 days, rats were killed, and about 2×10^8 spleen cells thereof were fused with 1×10^7 mouse myeloma cells P3V1 using 50% PEG1500 (Boehringer Mannheim) according to a conventional method, and hybridoma was selected in HAT medium.

[0027]

Hybridoma supernatant was added to an immunoplate coated with rabbit IgG antibody (Kappel) so as to react mouse soluble IL-6R, and the hybridoma producing an antibody to mouse soluble IL-6 receptor was selected by ELISA using rabbit anti-mouse IL-6R antibody and alkaliphosphatase-labeled goat anti-rabbit IgG antibody. Hybridoma confirmed to produce an antibody was twice subcloned to obtain a single clone (RR16-1).

[0028]

The neutralizing effects of antibodies produced by MR16-1 against IL-6 were tested by uptake of ^{3}H-thymidine by MH60.BSF2 cells (Matsuda, et al., Eur. J. Immunol. 18:951-956, 1988).

The MH60.BSF2 cells were distributed to a 96-well plate in the amount of 1×10^4 cells/200 ml/well, mouse IL-6 (10 pg/ml) and MR16-1 or RS12 antibody was added to the wells, and the cells were cultured at 37°C in 5% CO_2 for 44 hours. After that, ^{3}H-thymidine (1 mCi/well) was added to each well, and uptake of ^{3}H-thymidine was measured after 4 hours (Fig. 1).

[0029]

Example

Female day mice, 8 weeks old, were subjected to either a sham operation or ovariectomy (OVX), and sacrificed 2 weeks post-operatively. Bone marrow was isolated with phenol red-free αMEM from the tibia and the femora, and bone marrow cells were removed by centrifugation to obtain bone marrow supernatant. Bone resorbing activity of the bone marrow supernatant was measured by an organ culture system using fetal mouse long bone pre-labeled with ^{45}Ca. Fetal radii and ulnae were prelabeled with ^{45}Ca by subcutaneously injecting $^{45}CaCl_2$ into the mother mouse, then isolated from the fetus on day 17 of gestation. These were then cultured in a phenol red-free αMEM in 5% CO_2 (95% air) at 37°C.

[0030]

After 24 hours, the medium was replaced with a phenol red-free αMEM containing 0.2% BSA. At the same time, samples of bone marrow supernatant were added (at 40%). The bone resorption activity present in bone marrow supernatants from OVX mice was about 60%, while about 25% for sham mice.

Bone marrow supernatants from OVX mice were pre-treated at (37°C, 5% CO_2 for 2 hours) with 66 μg/ml of anti-mouse IL-6 antibody or RS12 antibody (continuously added to the culture). Both of the antibodies suppressed the bone resorbing activity present in bone marrow supernatants from OVX mice (Fig. 2).

[0031]

Example 2

A co-culturing system comprising mouse osteoblasts and bone marrow cells was used as an assay for osteoclast formation (Takahashi, et al., Endocrinology 122: 1373, 1988; Takahashi et al., Endocrinology 123: 2600, 1988). This assay served as a measure of bone resorption.

[0032]

The mouse osteoblasts were prepared as follows. Clavaria were removed aseptically from 1 or 2 day-old ddy mice and put into PBS-containing 0.1% collagenase (Wako Pure Chemical) and 0.2% dispase (Godo Shusei) to disperse the cells, which were then shaken at 37°C for 10 minutes. Suspended cells were collected, and fresh enzyme solution was added for enzyme treatment for 10 minutes. This enzyme digestion was repeated five times, and cells which were suspended by the 2nd to 5th digestions were recovered as mouse primary osteoblasts.

[0033]

Mouse bone marrow cells were prepared as follows. The tibiae were aseptically removed from 6 to 9 week-old ddy mice and the ends of the tibiae were cut off. 1 ml of α-minimum essential medium (α-MEM; GIBCO) was injected to the distal end of the tibia and the medium was recovered from the proximal end of the tibia. The cells were suspended in α-MEM containing 10% fetal calf serum (Bio Cell).

[0034]

For the co-culture, 1×10^4 cells/0.5 ml/well of osteoblasts and 2×10^5 cells/well of bone marrow cells were added to a 48-well culture plate and cultured for 6 - 7 days. Osteoclasts thus formed were identified by staining tartarate-resistant phosphatase (TRAP), which is a marker enzyme for osteoclasts (Takahashi, et al., Endocrinology 122, 1373 1988).

[0035]

To these culture systems, mouse IL-6 or mouse sIL-6R (IL-6sR(sR324); Saito, J. Immunol., 147: 168 - 173, 1991), and mouse IL-6R antibody (MR16-1 or RS12) or mouse IL-6 antibody (R&D Systems), were added. Note, for mouse IL-6 and sR324, supernatants of CHO cells transformed with a gene coding for one of the above-mentioned proteins were used. Concentrations of mouse IL-6 and sR324 in the culture supernatant were measured by enzyme immunoassay, and were used for further

experiments after dilution with α-MEM to an appropriate concentration.

[0036]

Experiment 1 Formation of osteoclasts by mouse IL-6 or mouse sIL-6R alone

The formation of osteoclasts in mouse IL-6 (0.2 ng to 200 ng/ml) or sR324 (0.5 to 500 ng/ml) alone was tested. Each protein alone did not promote osteoclast formation. Note that in this experimental system, an active type of vitamin D_3 as a positive control exhibited significant osteoclast formation activity (Fig. 3).

[0037]

Experiment 2 Formation of osteoclasts with a combination of mouse IL-6 and mouse sIL-6R

Osteoclast formation with a combination of 20 ng/ml or 200 ng/ml of mouse IL-6 and 0.05 to 500 ng/ml of sR324 was tested. Remarkable concentration-dependent osteoclast formation was observed (Fig. 4). Note that when these osteoclasts were cultured on dentine slices, many pit formation was observed and the formation of the resorption pits was inhibited by addition of calcitonin. This is an indication that the osteoclasts formed in this experimental system are mature osteoclasts having bone resorbing ability and calcitonin receptors.

[0038]

Experiment 3 Inhibitory effects of anti-mouse sIL-6R antibody (MR16-1 or RS12) or anti-mouse IL-6 antibody on osteoclast formation induced by a combination of mouse IL-6 and mouse sIL-6R

When osteoclast formation was tested in the presence of 20 ng/ml or 200 ng/ml of mouse IL-6 and 62.5-500 ng/ml of sR324, similar to Experiment 2, remarkable concentration-dependent osteoclast formation was observed. MR16-1, at a

concentration of 1 ng/ml to 100 ng/ml, inhibited osteoclast formation induced by 20 ng/ml of mouse IL-6 and 500 ng/ml of sR324 in a concentration-dependent manner. RS12 and anti-mouse IL-6 antibody showed inhibitory effects on osteoclast formation at 10 ng/ml and 100 ng/ml (Fig. 5).

[0039]

[Effects of Invention]

As demonstrated by the above, the therapeutic composition of the present invention, comprising as an effective ingredient an anti-sIL-6R antibody, inhibits osteoclast formation induced by IL-6 and sIL-6R, and bone resorbing activity in organ culture systems. Accordingly, the present invention is promising for the treatment of various diseases associated with bone resorption, such as osteoporosis, chronic rheumatoid arthritis, multiple myeloma, tumor-associated hypercalcemia, renal osteodystrophy, Paget's disease, osteocarcinoma and bone tumor metastasis.

[BRIEF DESCRIPTION OF DRAWINGS]

[Fig. 1]

Figure 1 is a graph which represents the effects of the antibody MR16-1 or RS12 on IL-6-dependent growth of MH60.BSF2 cells.

[Fig. 2]

Figure 2 is a graph which represents the neutralization effects of antibody on bone resorption activity of bone marrow supernatants collected from OVX mice (2 weeks).

[Fig. 3]

Figure 3 is a graph which shows the effects of IL-6 or soluble IL-6R (sR324) on osteoclast formation in co-cultures of mouse osteoblasts and bone marrow cells.

[Fig. 4]

Figure 4 is a graph which shows the effects of

combinations of both IL-6 and sR324 on osteoclast formation in co-cultures of mouse osteoblasts and bone marrow cells.

[Fig. 5]

Figure 5 shows the inhibitory effects of anti-IL-6R antibody on osteoclast formation in co-cultures of mouse osteoblasts and bone marrow cells in the presence of both IL-6 and soluble IL-6R (sR324).

[NAME OF DOCUMENT] ABSTRACT

[SUMMARY]

[PURPOSE] To provide a novel agent for inhibition of bone resorption.

[CONSTITUTION]

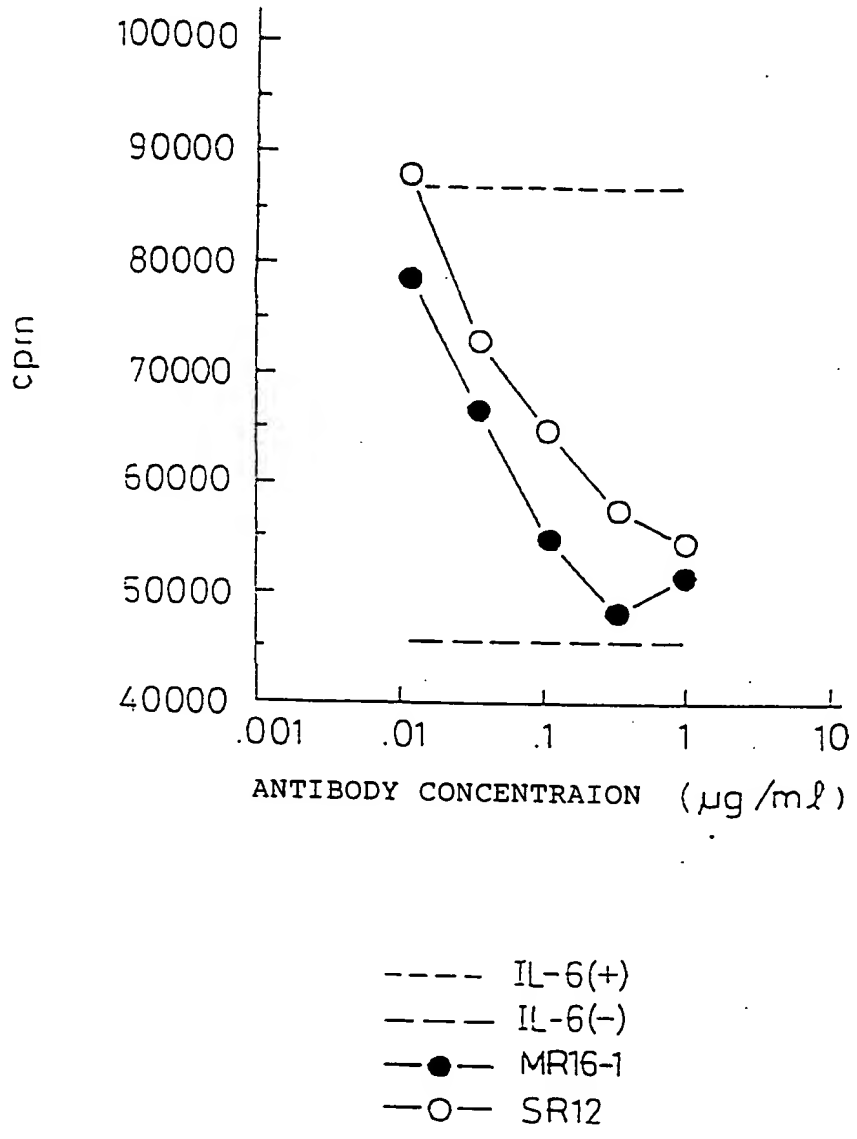
A pharmaceutical composition for inhibition of bone resorption comprising an antibody to interleukin-6 receiptor.

[EFFECT] If efficiently inhibits bone resorption action due to inhibition of formation of osteoclasts in the presence of both of interleukin 6 and interleukin 6 receptor, and is effective as therapeutic agent for various diseases involving in bone resorption.

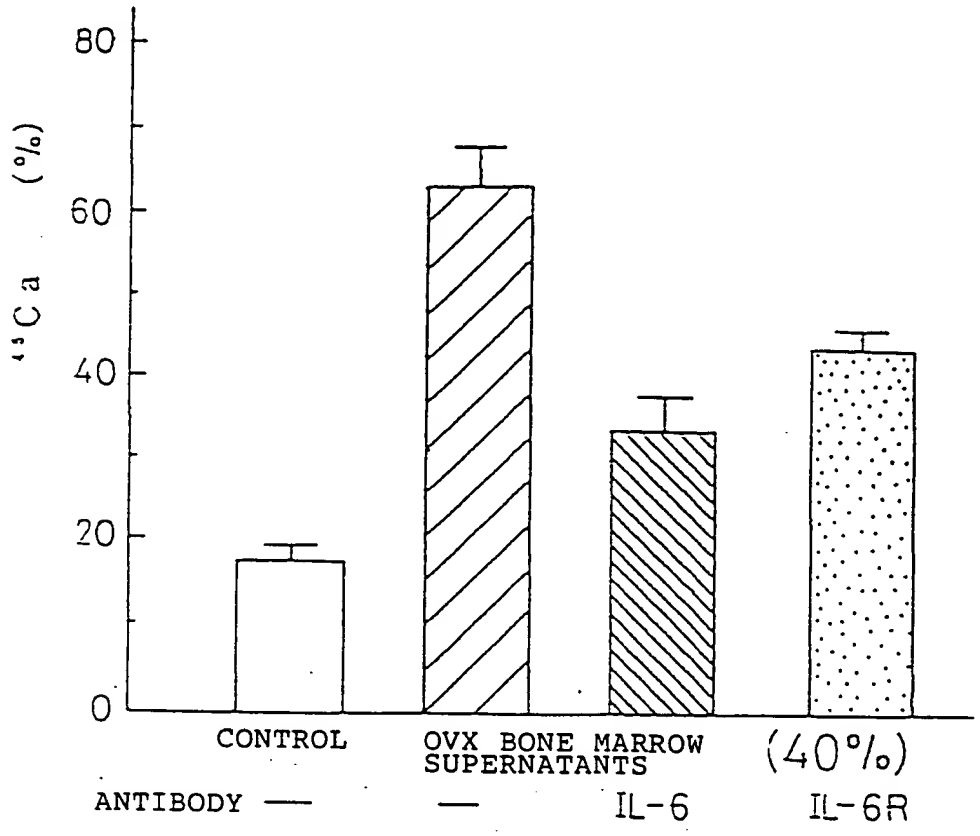
[REPRESENTATIVE DRAWING] Fig. 5

[NAME OF DOCUMENT] DRAWING

[Fig.1]



[Fig.2]


80
60
40
20
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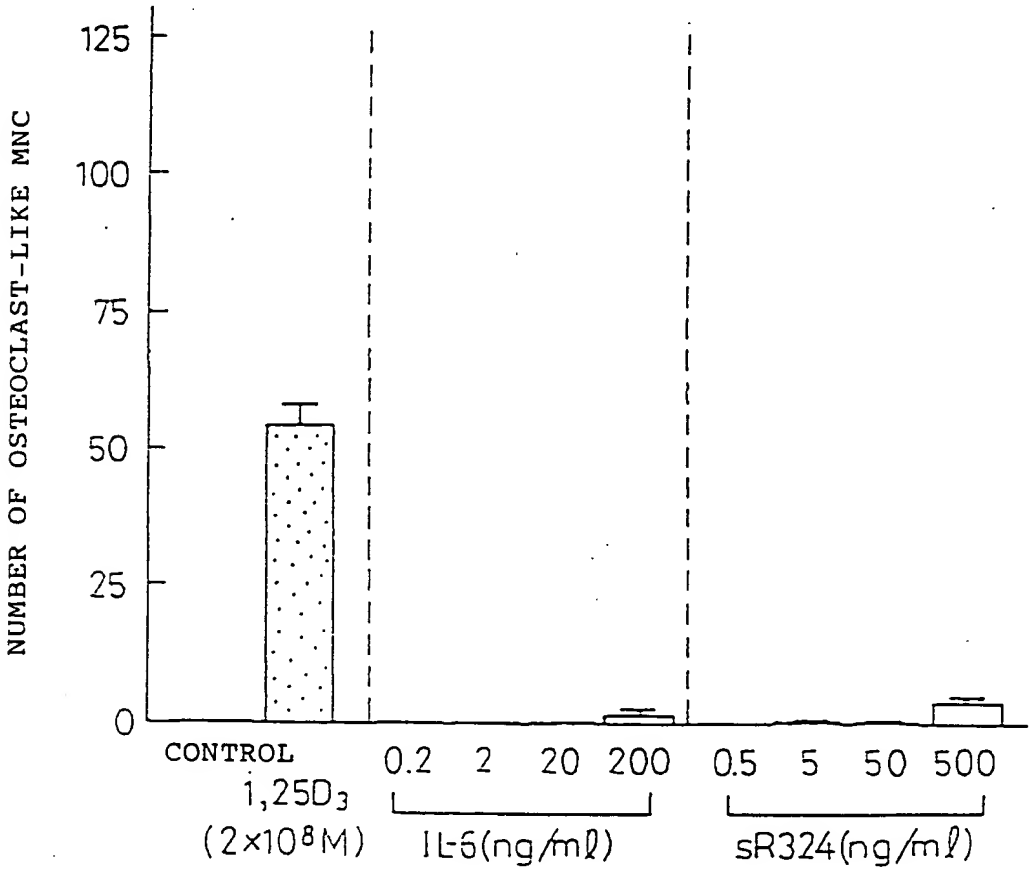
(40%)
ANTIBODY —
—
IL-6
IL-6R

[Fig.3]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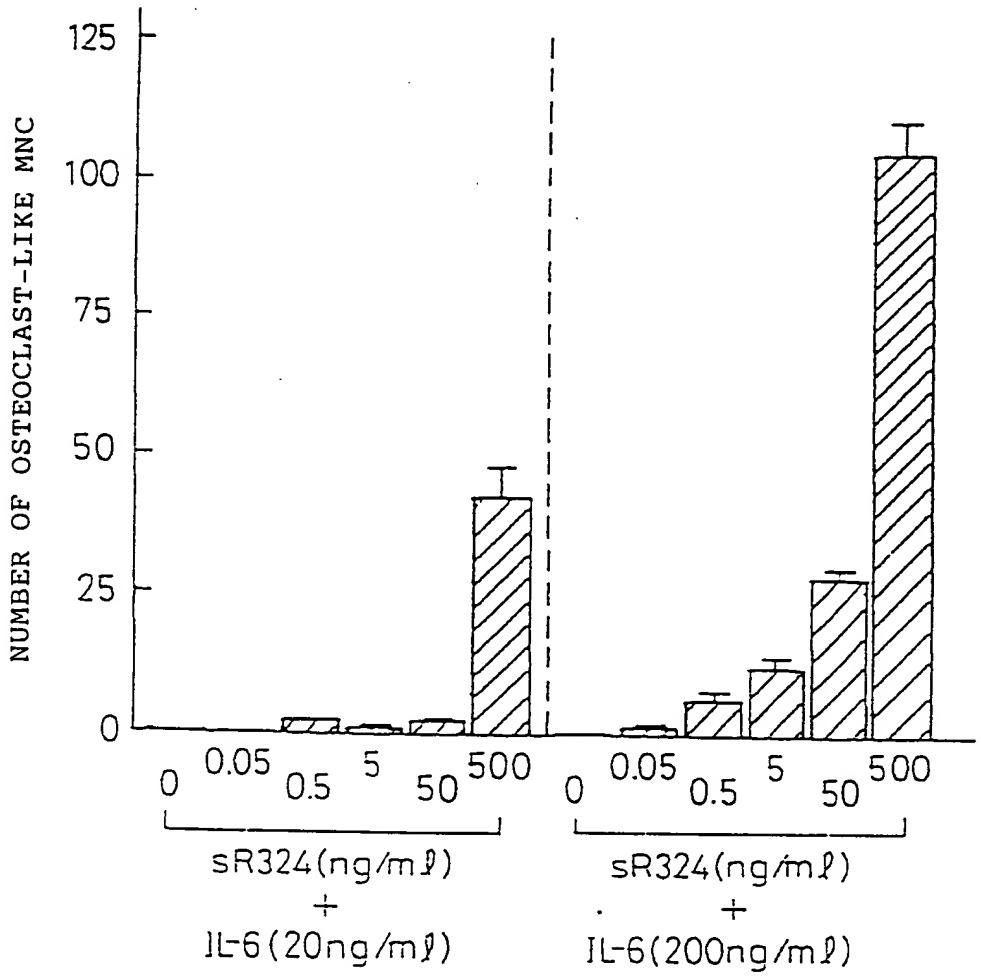
0.5 5 50 500
sR324(ng/mℓ)

[Fig.4]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0.5
5
50
500
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IL-6(20ng/mℓ)
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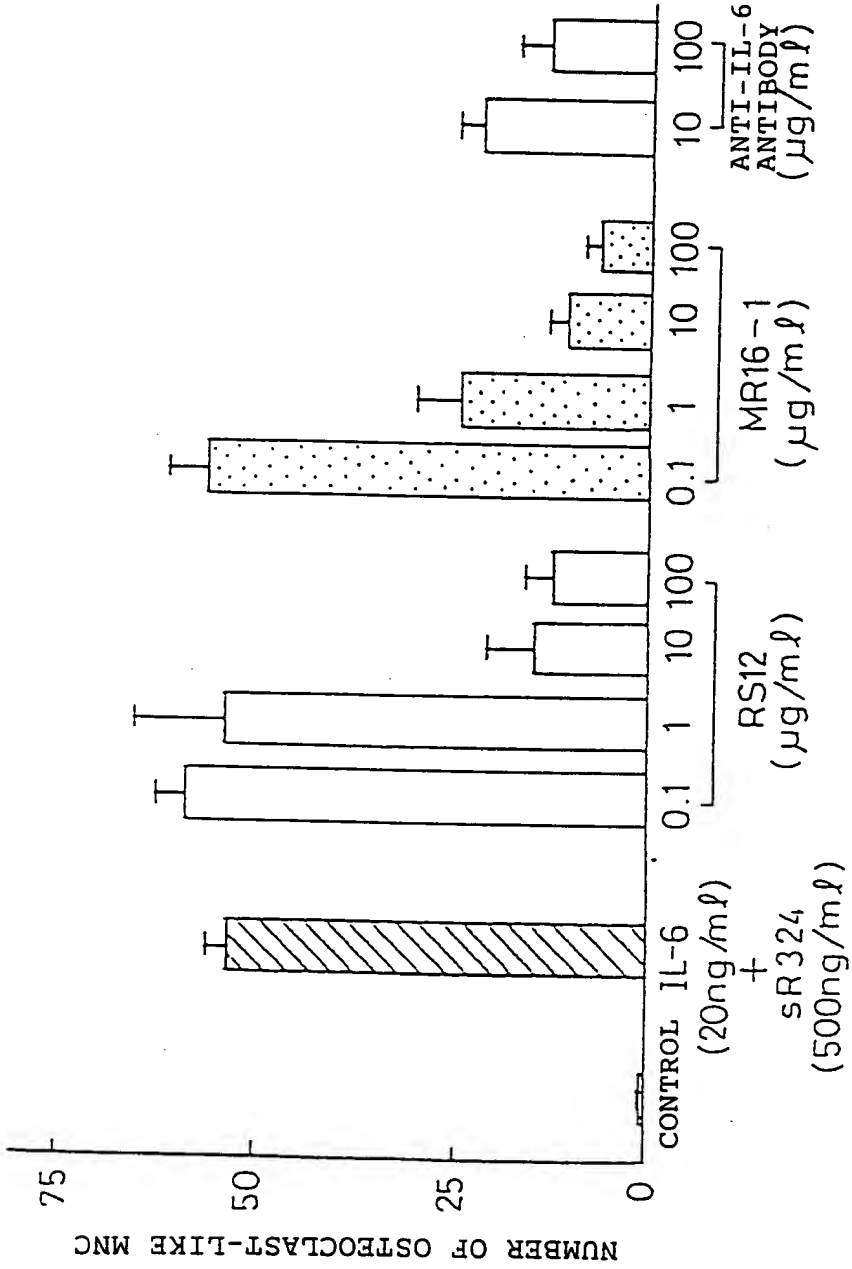
+
IL-6(200ng/mℓ)

[Fig.5]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100
RS12 (μg/mℓ)
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1
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MR16-1 (μg/mℓ)
10
100
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